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Urinary excretion of neutral proteinases in nephrotic rats with a glomerular disease

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Urinary excretion of neutral proteinases in nephrotic rats with a glomerular disease. A proliferative glomerulonephritis was induced in rats pre-immunized with rabbit IgG by injecting intravenously a sub-nephrotoxic dose of rabbit anti-glomerular basement membrane (GBM) IgG (A rats). Most rats (80%) developed a severe proteinuria (>100 mg/24 hr) within two to five days after the injection of anti-GBM IgG. At the same time, microscopic examination of the kidneys revealed a glomerular infiltration by mononuclear phagocytes and a prominent decrease in the intensity of the colloidal iron reaction in glomeruli. A non-proliferative glomerular disease was induced in another group of rats (B rats) by intraperitoneal administration of aminonucleoside of puromycin. A marked proteinuria (>100 mg/24 hr) occurred after six days in 90% of animals. Histochemical studies then revealed a decrease in staining intensity of glomeruli for polyanion. No glomerular hypercellularity was noted. In normal rats and in non-proteinuric A or B rats, the 24 hour urinary excretion of neutral proteinases ranged from 1.4 to 7.8 units (mean value ± SEM, 4.69 ± 0.60, $N = 11$), that of laminin ranged from 100 to 3,900 ng (mean value ± SEM, 1,154 ± 325, $N = 10$), and that of type IV collagen ranged from 160 to 420 ng (mean value ± SEM, 306 ± 26.5 ng, $N = 8$). In proteinuric rats from groups A ($N = 11$) and B ($N = 9$), the 24 hour urinary excretion of neutral proteinases significantly increased (mean values ± SEM, 38.55 ± 8.66 U for A rats and 42.17 ± 7.92 U for B rats) and ran parallely with that of proteins, laminin and type IV collagen. Significant direct linear correlations were observed between: (a) the urinary neutral proteinase activity and the proteinuria, the urinary excretion of laminin and that of type IV collagen; and (b) the proteinuria and the urinary excretion of laminin. In A and B rats, as in normal rats, proteinase activity occurred in urine as an active form exhibiting an apparent molecular weight of 30,000 and an isoelectric point of 9.0, was maximal in the neutral range, was eliminated by heating at 60°C for 30 minutes and was found capable of degrading GBM polypeptides (laminin and type IV collagen) in vitro. In normal urine, a significant inhibition of the neutral proteinase activity was seen with phenylmethylsulfonylfluoride, soybean trypsin inhibitor or aprotinin, but not with EDTA or cysteine, which suggests that the enzymatic activity mainly results from (a) serine proteinase(s). In urine from A or B rats, neutral proteinase activity was significantly reduced by all the inhibitors tested which indicates the presence of both (a) serine proteinase(s) and (a) metallo-proteinase(s). Our in vitro and in vivo findings strongly suggest that in these two experimental models of glomerular disease, neutral metalloproteinases may be involved in the damage to the GBM and in the development of proteinuria.

The impermeability of the glomerular filtration barrier to anionic proteins is partly due to its electronegative charges, as suggested by the following observations: (1) polycations selectively bind to the glomerular epithelial cells, their pedicelles, the endothelial cell coat and the laminae rarae [1–4]; (2) this binding is decreased in experimental models of proteinuria such as the aminonucleoside nephrosis (AN) [5, 6] and the nephrotoxic nephritis (NTN) [7]; (3) the filtration fraction of cationic dextrans is higher than that of anionic dextrans in normal rats [8]; and (4) the filtration fraction of anionic dextrans is higher in AN and NTN rats than in normal rats [6, 9].

The biochemical knowledge of the electronegative molecules present in the glomerular filtration barrier is in progress. It has been shown [10] that sialic acid is one of these molecules, since neuraminidase infusion into rats induces a loss of anionic charges of epithelial and endothelial cells [10]. The same authors have also demonstrated that GBM glycosaminoglycans play a major role in the restricted transport of polyanions across the glomerular capillary wall [11].

The ability to obtain reliable growths of glomerular cells in culture has allowed the study of the biosynthesis and degradation of GBM components in vitro. It has been shown [12–15] that the epithelial and mesangial cells contribute to the biosynthesis of type IV collagen, non-collagenous glycoproteins and glycosaminoglycans. Neutral proteinases generated by cells derived from the glomerular mesangium are capable of degrading GBM glycoproteins in vitro [16]. In immunological glomerulonephritis (GN), monocytes and neutrophils frequently infiltrate the glomerulus [17, 18]. These cells also contain neutral proteinases which extensively degrade the GBM at physiological pH [19, 20]. These findings suggest that neutral proteinases may be implicated in the physiological turnover and in the increased degradation of GBM anionic proteins in vivo.

In this work, we have first studied the urinary neutral proteinase activity and the urinary excretion of GBM components, that is, laminin and type IV collagen, in normal rats. We have then measured the same parameters in the urine from rats presenting with a glomerular disease and a severe proteinuria in order to provide a support to the concept that neutral proteinases may play a role in the damage to the GBM. Since neutral proteinases can be generated in vitro by intrinsic glomerular cells as well as by inflammatory cells, we have also compared the urinary excretion of these enzymes during the course of two experimental models of glomerular diseases, that

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is, a non-proliferative glomerular disease and a GN associated with an infiltration of glomeruli by mononuclear phagocytes.

Methods

The experimental model of nephrotoxic nephritis (NTN)

Nephrotoxic serum was prepared as previously described [21, 22]. Briefly, rabbits were immunized by intramuscular injection of 5 mg of lyophilized GBM emulsified in complete Freud's adjuvant (Difco Laboratories, Detroit, Michigan, USA). One injection was given each week for three weeks, followed by a single injection three weeks later. The rabbit serum was absorbed for hemagglutinins and decomplemented. IgG fraction was isolated by passage through DEAE-cellulose columns [23]. The presence and specificity of anti-GBM IgG were demonstrated by radioimmunoassay [22] and by indirect immunofluorescence performed on normal rat kidney slices [24]. A linear deposition of IgG was observed along the GBM, the tubular basement membranes and the Bowman's capsules. Sprague Dawley (female) rats weighing 100 to 120 g were pre-immunized with 1 mg of rabbit IgG in 0.5 ml of complete Freud's adjuvant injected intraperitoneally. One week later, the rats received 1 mg of rabbit anti-GBM IgG intravenously. This dose was chosen on the basis of preliminary experiments demonstrating that 1 to 2 mg of rabbit anti-GBM IgG did not induce a significant proteinuria in non-preimmunized rats [25].

The experimental model of aminonucleoside of puromycin nephrosis (PAN)

A nephrotic syndrome was induced in Sprague Dawley rats weighing 100 to 120 g by the administration of aminonucleoside of puromycin (Sigma Chemical Co., St. Louis, Missouri, USA) in a single intraperitoneal injection of 150 mg per kg. Animals were sacrificed three to 24 days after administration of aminonucleoside [26].

Assessment of urinary neutral proteinase activity

Methods described in the present paper have been extensively detailed [19, 27]. Briefly, 24 hour urine collections were exhaustively dialyzed at 4°C against distilled water, were sterilized by filtration through Millipore filters (Millipore Benelux, Brussels) and were then stored at −20°C until use. Neutral proteolytic activity against azocasein (Azocoll, Calbiochem–Behring Corp., La Jolla, California, USA) or ^{3}H-labelled acetylcasein was determined on 0.2 ml urine samples according to the method described by Starkey [27], except that a temperature of 45°C was used [19]. ^{3}H-labelled acetylcasein was used as a substrate for screening purposes, whereas the precise measurement of the urinary enzyme activity was performed using azocasein as a substrate. Under the conditions of the assay, the enzyme activity was linear with time and with enzyme concentration. Enzyme activity is expressed in units per ml, where one unit is defined as the amount of enzyme that will hydrolyze 1 mg of substrate per hour. In order to identify which enzyme was responsible for the degradation of substrates, experiments with various inhibitors of tissue proteinases were undertaken [16, 20]. The following inhibitors were tested: EDTA 2 mM or 15 mM, cysteine 2 mM, soybean trypsin inhibitor (SBTI) (100 and 500 μg/ml), aprotinin (Trasylol) (500 μg and 1 mg/ml) and phenylmethylsulfonylfluoride (PMS-F) 1 mM. Enzyme activity was then expressed as the percentage of activity in the absence of inhibitors. Finally, the pH optimum of the neutral proteinase activity was determined by varying urine pH over a range of 6.0 to 8.5, and thermolability was assessed by heating urine samples to 60°C for 30 minutes before testing.

In order to study the effects of neutral proteinases on BM components, [^{14}C]-labelled type IV collagen or [^{3}H]-laminin were prepared as previously described [28, 29]. The purity of the preparations was assessed by polyacrylamide slab gel electrophoresis [14]. Five μg of labelled BM polypeptides (about 5,000 cpm) were added to 0.2 ml of 0.02 M Tris-HCl buffer pH 7.6, containing 10 mM $CaCl_2$, 0.05% NaN_3, penicillin (100 U/ml) and streptomycin (100 μg/ml). Dialyzed urine samples (0.2 ml) were then incubated with the labelled BM glycoproteins for 48 hours at 37°C. At the end of the incubation, the samples were exhaustively dialyzed against distilled water and their radioactivity was counted in a Packard Tricarb Counter after addition of 10 ml of Bray's solution [29]. Controls included BM glycoproteins added to buffer alone (0.4 ml) or to urine samples (0.2 ml) supplemented with PMS-F 1 mM and EDTA 2 mM. Each experiment was done in triplicate. Results are expressed as the percentage of the radioactivity counted in control samples. Enzyme inhibition profiles were obtained using the same conditions as described above for azocaseinase. Gel chromatography for the determination of apparent molecular weights was performed using a 1.5 × 60 cm Sephacryl S-200 SF column (Pharmacia, Uppsala, Sweden) [16]. Isoelectric points were determined with the chromatofocusing kit provided by Pharmacia Fine chemicals. Columns (1.6 × 40 cm, Pharmacia) were packed with PBE 94 gel. The start buffers used were 0.025 M ethanolamine-CH_3COOH pH 9.4 for the pH range from 9 to 6 and 0.025 M imidazole-HCl pH 7.4 for the pH range from 7 to 4; the polybuffers 96-CH_3COOH pH 6 and 74-HCl pH 4.0 were used as eluents for the former and the latter pH range, respectively.

Assessment of proteinuria and of urinary laminin or type IV collagen excretions

Urine was collected at 24 hour intervals and protein concentrations were measured by the method of Kingsbury and Clarck [30]. The urinary excretion of laminin and of type IV collagen was also determined on 24 hour urine collections by a solid phase radioimmunoassay using affinity purified, anti-laminin or anti-type IV collagen rabbit antibodies and ^{125}I-protein A as a tracer [31, 32]. The specificity of these antibodies and the reproducibility of radioimmunoassays have been previously described [31, 32]. Standard curves were established by mixing 10 to 1,000 ng of either laminin or type IV collagen to 1 ml of phosphate buffered–saline, pH 7.4. Results were expressed in ng per 24 hours.

Morphological studies of the kidney sections

The kidney were examined by light and immunofluorescence microscopy at various periods of time, from three to 21 days, after the injection of anti-GBM IgG or of aminonucleoside. For light microscopy, portions of rat kidneys were fixed in Bouin's renal fixative, sectioned at 4 μm and stained with hematoxylin and eosin, periodic acid–Schiff reagent and with colloïdal iron at

pH 1.9 [5]. Kidney specimens were also snap frozen in liquid nitrogen, sectioned at 2 μm on a cryostat and stained with fluorescein isothiocyanate (FITC) conjugated antisera to rabbit IgG, rat Ig and rat C3. Commercial FITC-antisera were used (Behring–Werke AG, Marburg/Lahn, West Germany). The kidney sections were also incubated for 30 minutes at room temperature with fluorescein–conjugated rabbit IgG directed against laminin or type IV collagen. The specificity of these antibodies has been previously demonstrated [33].

Results

The experimental model of NTN (A rats)

The morphological aspect of glomeruli within two to five days after the injection of anti-GBM IgG has been previously described [25]. Briefly, microscopic examination of the kidneys revealed a glomerular hypercellularity which was the consequence of an infiltration by mononuclear cells and a proliferation of intrinsic glomerular cells. No crescents were seen. After two to three weeks, there was a progressive thickening of the GBM and a simultaneous decrease of the hypercellularity [25]. Immunofluorescence study of GN kidneys demonstrated the presence of linear deposits of rat Ig, rat C3 and rabbit IgG along the GBM. No fixation was detectable on other kidney structures [25].

The experimental model of PAN (B rats)

In this model, no glomerular hypercellularity was observed; as others [5, 26], we only noted multiple small eosinophilic droplets in glomerular epithelial cells and eosinophilic hyaline casts in some Bowman's spaces and tubular lumens. Some proximal convoluted tubules showed epithelial fragmentation and collapse. These changes were particularly obvious between five and 12 days after PAN injection. By 21 days, glomeruli and tubules were indistinguishable from those of normal rats. At the same time, PAN kidney specimens were not significantly different from those of normal rats with respect to the presence of IgG and C3. A slight increase in staining for IgM in an indeterminate pattern was however observed in glomeruli from PAN rats (data not shown).

Proteinuria

Control values for total 24 hour urinary protein excretions in the rats used in this study did not exceed 10 mg. Rats injected with 1 to 2 mg of rabbit anti-GBM IgG did not develop a pathological proteinuria. Pre-immunization with 1 mg of rabbit IgG seven days before the injection of 1 mg of anti-GBM IgG induced a severe proteinuria starting on day two and reaching maximum values on day four. The proteinuria decreased thereafter to reach about 50 mg/day three weeks later (Fig. 1A). Figure 1B shows the evolution of the 24 hour urinary protein excretions following the injection of PAN. A proteinuria appeared after a delay of six days, increased to a maximum value of 180 mg per 24 hours on day 12 and progressively declined to reach near–normal values by 22 to 24 days. The mean proteinuria (± SEM) was 240 ± 23 mg/24 hours on day four in A rats (range, 110 to 420 mg/24 hr, $N = 11$), whereas it was 180 ± 31 mg/24 hours on day 12 in B rats (range, 95 to 310 mg/24 hr, $N = 9$). Both groups of rats therefore developed a severe and comparable proteinuria.

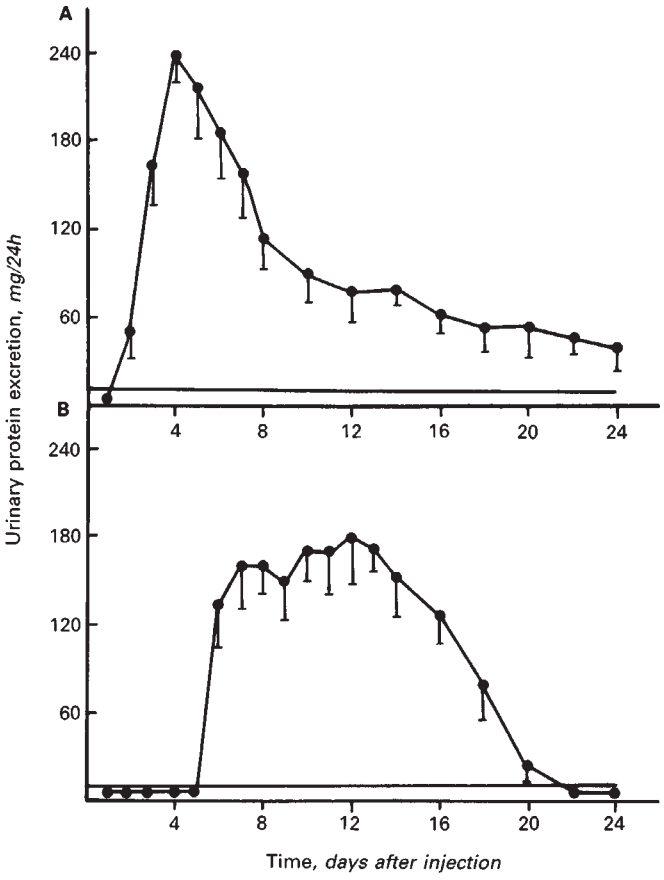

Fig. 1. *Evolution of the proteinuria in rats injected with anti-GBM IgG* **(A)** *or in rats injected with aminonucleoside* **(B).** Values of proteinuria are expressed in mg par 24 hr (±SEM). In each group, proteinuria was measured in eight rats at least.

Urinary neutral proteinase activity

Proteinase activity against azocasein, ^{3}H-labelled acetylcasein, was present in the urine from normal, A or B rats. This enzymatic activity was destroyed by heating at 60°C for 30 minutes and was maximal in the neutral range. Less than 60% activity were indeed detectable below pH 7.0 or above pH 8.0 (data not shown). In nine normal rats, the urinary neutral proteinase activity ranged from 1.4 to 7.8 U/24 hours (mean value ± SEM: 4.69 ± 0.60) (Fig. 2). In 11 NTN rats tested four days after the injection of anti-GBM IgG, the urinary neutral proteinase activity was strongly increased (mean value ± SEM, 38.55 ± 8.66 U/24 hr, range, 12.3 to 105.6 U/24 hr, Fig. 2). In B rats, the urinary neutral proteinase activity measured on day 12 was 42.17 ± 7.92 U/24 hours (range, 15.92 to 83.7 U/24 hr, $N = 9$, Fig. 2). The differences observed between the urinary neutral proteinase activity of normal and A rats, and between that of normal and B rats, were all statistically significant ($P < 0.001$). The urinary neutral proteinase activity was sequentially measured in four rats from group A and in two rats from group B. It was found that the 24 hour urinary excretion of neutral proteinases was maximal on day four for A rats and on day 12 for B rats, as that of proteins (data not shown).

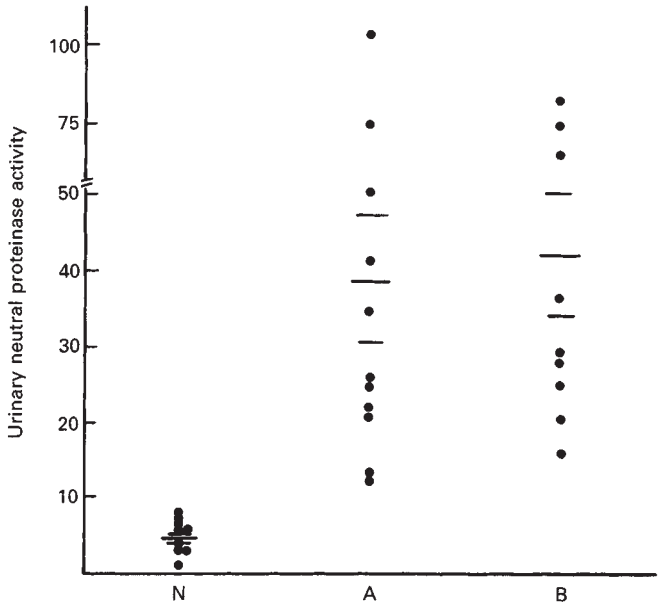

Fig. 2. *Urinary neutral proteinase activity in nine normal rats (N), 11 NTN rats (A) on day four and nine PAN nephrosis rats (B) on day 12.* Results are expressed in units (U) per 24 hr. One unit is the amount of enzyme hydrolyzing 1 mg of substrate per hour. Horizontal lines indicate means ± SEM. A and B values are significantly higher than N values ($P < 0.001$).

Urinary excretion of laminin and type IV collagen

In normal rats, the urinary laminin excretion was 1,154 ± 325 ng/24 hours (mean value ± SEM, range 100 to 3,000 ng/24 h, $N = 10$, Fig. 3), whereas the urinary type IV collagen excretion was 306 ± 36.5 ng/24 hours (mean value ± SEM, range, 160 to 420 ng/24 hr, $N = 8$, Fig. 3). In A rats tested four days after the injection of anti-GBM IgG, the mean urinary excretion of laminin (±SEM) was 12,371 ± 2,205 ng/24 hours (range, 6,900 to 38,400 ng/24 hr, $N = 11$, Fig. 3). In B rats, the mean urinary excretion of laminin (±SEM), measured 12 days after PAN injection, reached 15,878 ± 3,038 ng/24 hours (range, 8,100 to 38,800 ng/24 hr, $N = 9$, Fig. 3). The mean urinary laminin excretion per day was therefore significantly higher in nephrotic than in normal rats ($P < 0.001$). Comparable data were obtained for the urinary excretion of type IV collagen (Fig. 3). Finally, it was found that the urinary excretions of laminin or of type IV collagen in A and B rats were not statistically different. The urinary excretion of laminin and of type IV collagen was measured in four NTN rats and in two PAN rats on two different days after injection of either nephrotoxic serum or aminonucleoside. Figure 4 clearly shows that the 24 hour urinary excretion of both structural glycoproteins ran parallel with that of proteins (Figure 1).

In both groups of nephrotic rats, significant direct linear correlations were observed between the following parameters: (1) the proteinuria and the urinary proteinase activity (Fig. 5); (2) the proteinuria and the urinary excretion of laminin (Fig. 5); and (3) the urinary neutral proteinase activity and the urinary excretion of either laminin or of type IV collagen (Fig. 6).

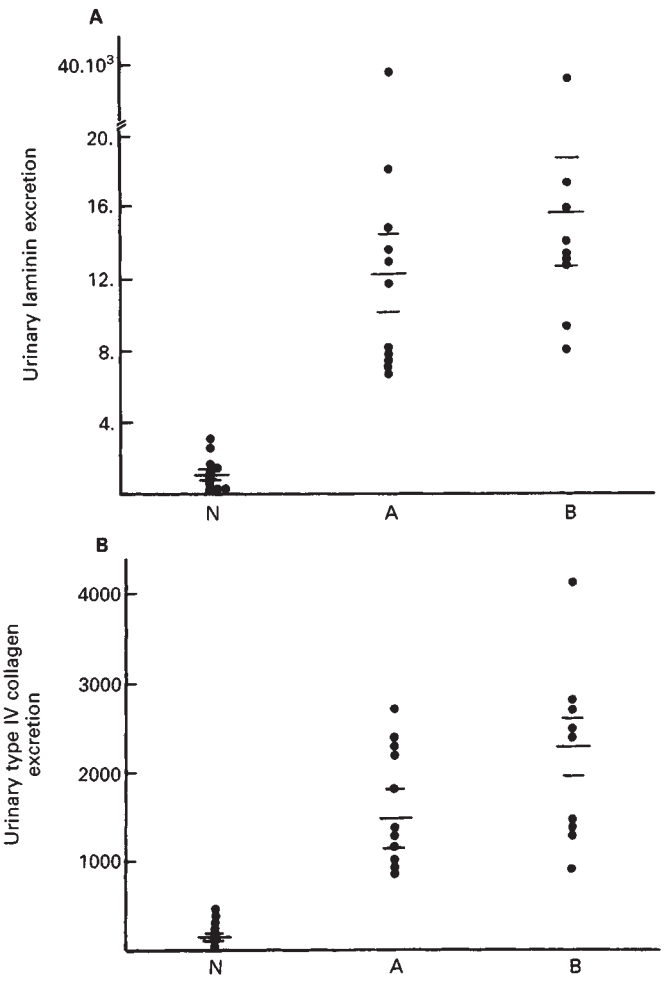

Fig. 3. A. *Urinary laminin excretion in 10 normal rats (N), 11 NTN rats (A) on day four and nine PAN rats (B) on day 12.* Results are expressed in nannograms/24 hr. Horizontal lines indicate means ± SEM. A and B values are significantly higher than N values ($P < 0.001$). **B.** Urinary type IV collagen excretion in seven normal rats (N), 11 NTN rats (A) on day four and nine PAN rats (B) on day 12.

Some properties of urinary neutral proteinases

In order to characterize the proteinase activity, urine samples from normal, A and B rats were preincubated with various proteolytic enzyme inhibitors (Table 1). In normal A and B rats, a marked inhibition of the enzymatic activity was obtained by the serine proteinase inhibitors (PMS-F, SBTI and aprotinin), whereas the metallo-proteinase inhibitors (EDTA and cysteine) inhibited the enzymatic activity in urine from A and B rats only. Gel chromatography of normal or nephrotic urine showed an active neutral proteinase activity peak with an apparent molecular weight of 30,000 daltons (Fig. 7). Furthermore, chromatofocusing experiments revealed that the isoelectric point of active enzymes in normal or nephrotic urine was about 9.0 (Fig. 8). Finally, the ability of urine from normal, A or B rats to degrade laminin and type IV collagen is shown in Table 2. In control urines, the degradation of both glycoproteins was lower than 10% after otherwise identical experimental conditions,

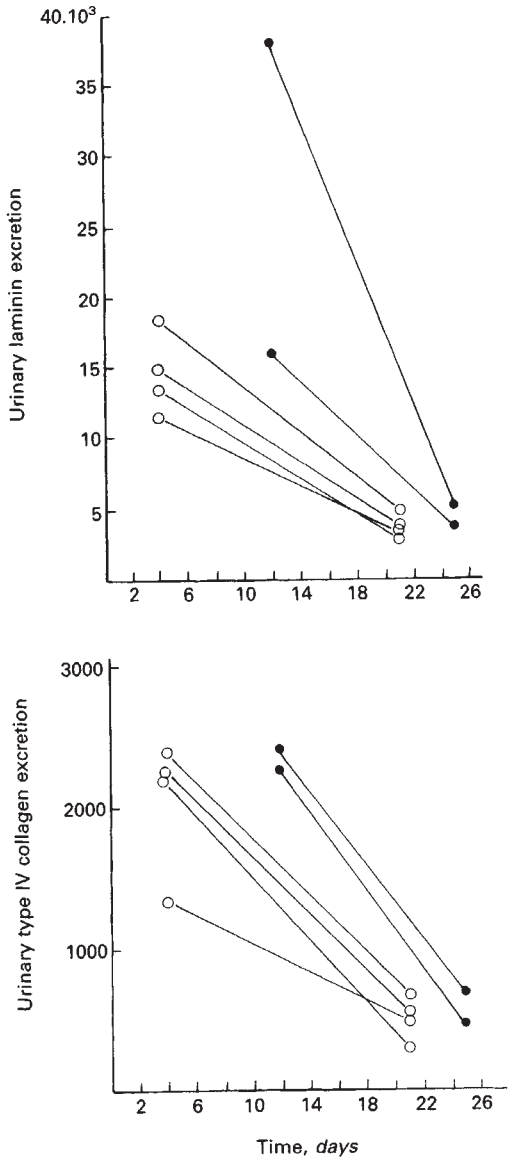

Fig. 4. A. Evolution of urinary laminin excretion in four NTN rats (○) and two PAN nephrosis rats (●). **B.** Evolution of urinary type IV collagen excretion in four NTN rats (○) and two PAN nephrosis rats (●).

which confirms the inhibitory properties of EDTA and PMSF on the urinary azocaseinase activity (Table 1).

Relation between the urinary neutral proteinase activity and the immunohistochemical studies of glomeruli

Sequential histochemical studies were conducted on kidney sections from NTN rats or from puromycin–treated animals at varying intervals after the nephrotoxic serum or the drug was administered. In each rat with a proteinuria and with a significant increase in the urinary neutral proteinase activity, a decrease and alteration in the staining intensity for glomerular polyanion was apparent when compared with kidney sections from normal rats (Table 3). Despite this fact, the pattern of staining and the intensity of binding to glomerular structures of antibodies directed against laminin (and type IV collagen) were not strongly altered in A or B rats, as shown by immunofluorescence microscopy (data not shown).

Discussion

Histochemists have long known that the glomerulus is rich in anionic sites for it stains with cationic reactants as colloidal iron and alcian blue [34, 35]. Chang et al [8] and Rennke, Cotran and Venkatachalam [1] have demonstrated the functional role for these anionic sites, the former by showing that the glomerular clearance of negatively charged dextrans was reduced when compared to that of neutral dextrans of the same size, and the latter by determining that the penetration of cationized ferritin molecules in the GBM was deeper than that of neutral or anionic ferritin molecules of the same size. Subsequently, it has been shown [4, 36, 37] by biochemical analysis and by enzyme digestion studies that the anionic sites of the GBM are localized in the laminae rarae interna and externa and mainly consist of sulfated glycosaminoglycans, such as heparan sulfate. Sialic acid present at the surface of endothelial and epithelial cells and in GBM glycoproteins (laminin, for example) is another polyanion of the glomerular filtration barrier [10]. Its removal by neuraminidase perfusion of rat kidneys determine a disappearance of the electronegative charges present at the surface of endothelial and epithelial cells and a detachment of those cells from the GBM, similar to that seen in aminonucleoside nephrosis [10].

In diseased states, the loss of electronegative charges from the glomerular filtration barrier have been demonstrated by clearance studies of variously charged dextrans or by histochemical analysis performed either in rats presenting with a nephrotoxic nephritis or an aminonucleoside nephrosis [5, 9, 38], or in patients presenting with an acquired or a congenital nephrotic syndrome [39, 40]. Michael, Blau and Vernier [5] have noticed in the aminonucleoside nephrosis of rats that the decrease in the glomerular sialoprotein content coincides with the onset of proteinuria and the foot–process fusion. Vernier et al [40] have suggested that congenital nephrosis results from failure of heparan sulfate–rich anionic sites to normally develop in the lamina rara externa of the GBM. More recently, Kerjaschki, Vernillo and Farquhar [41] have demonstrated that the sialic acid content of podocalyxin, the major sialylated rat glomerular protein, is reduced by 30% in aminonucleoside nephrosis.

In the present work, we have demonstrated that in rats presenting with a severe proteinuria: (1) the urinary laminin excretion is strongly increased; (2) this increase is temporally correlated with the loss of the glomerular colloidal iron staining at pH 1.9 [5]; and (3) the urinary excretion of laminin runs parallel with that of type IV collagen, a glycoprotein located, as laminin, in the GBM and in other basement membranes. The plasma levels of laminin and type IV collagen were not measured in the present work. One cannot therefore exclude that some laminin and type IV collagen antigens found in urine might come from other basement membranes than the GBM. However, puromycin aminonucleoside and anti-GBM antibodies primarily cause glomerular damage [5, 18]. Accordingly, we suggest that in both groups of rats, laminin molecules are well removed from the glomerular filtration barrier. The absence of

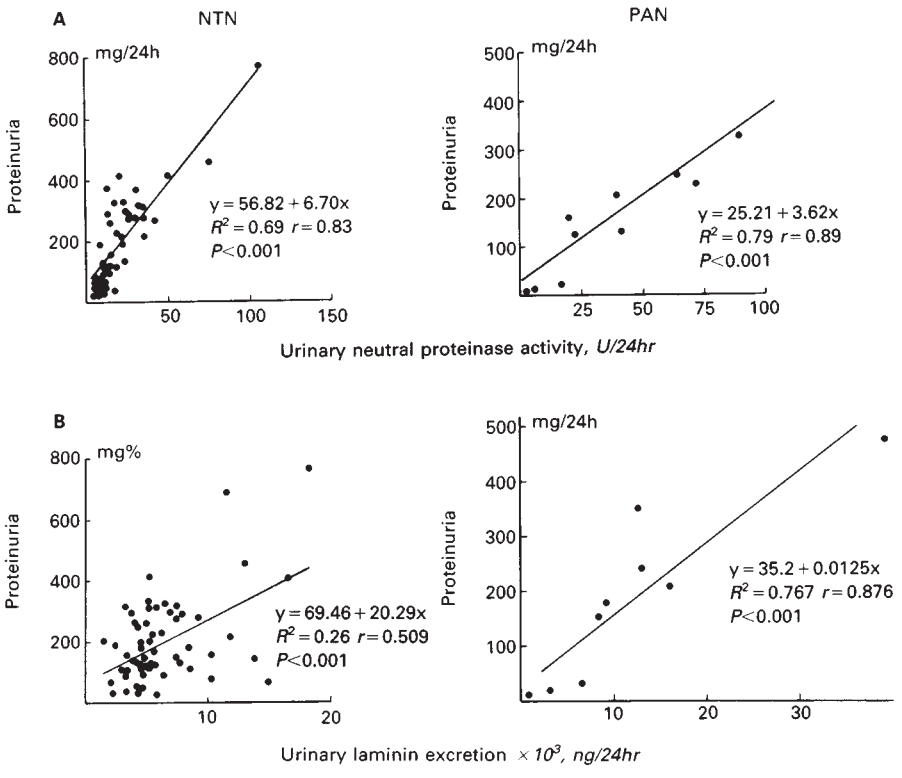

Fig. 5. A. *Correlation between the proteinuria and the urinary neutral proteinase activity in NTN and in PAN rats.* **B.** Correlation between the proteinuria and the urinary laminin excretion in NTN and PAN rats. All correlations are statistically significant ($P < 0.001$).

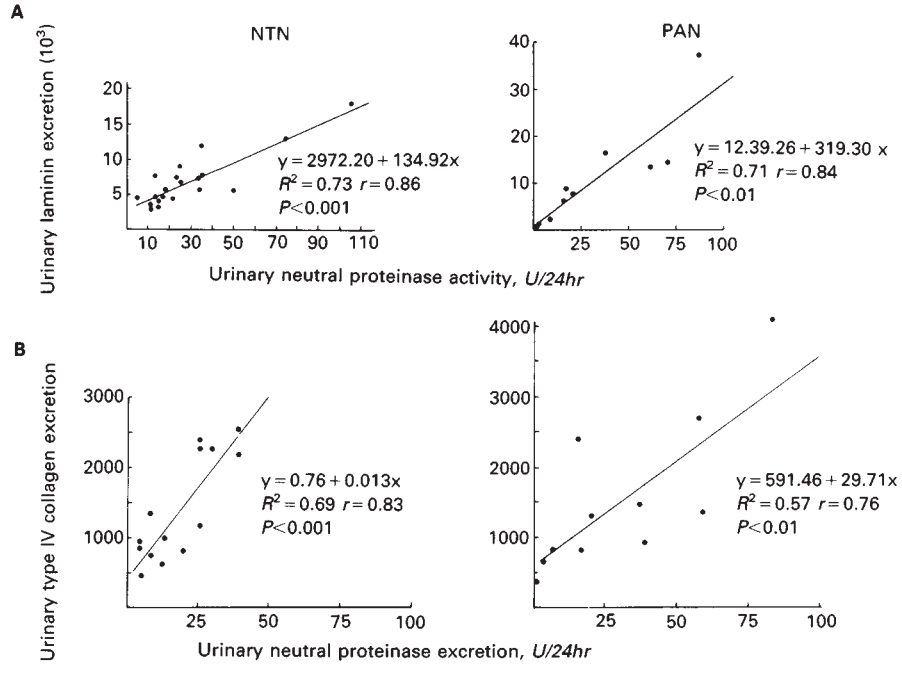

Fig. 6. A. *Correlation between the urinary laminin excretion (ng/hr) and the urinary neutral proteinase activity in NTN (A) and PAN (B) rats.* These correlations are statistically significant $P < 0.001$ and $P < 0.01$, respectively. **B.** Correlation between the urinary type IV collagen excretion (ng/hr) and the urinary neutral proteinase activity in NTN and PAN rats. These correlations are statistically significant, $P < 0.001$ and $P < 0.01$, respectively.

a clear cut alteration of the binding of anti-laminin antibodies to the GBM of diseased kidneys, as observed by immunofluorescence microscopy, does not really argue against this interpretation since it is well known that the definition of immunofluorescence techniques is rather poor. Ultrastructural studies of kidney sections incubated with anti-laminin antibodies coated to gold particles are now being pursued to clarify this point. Further studies will be also necessary to know whether the loss of laminin molecules is important or not in the overall electro-negativity of the GBM.

Several works [16, 19, 42] have shown that neutral proteinases generated by mesangial cells, monocytes and neutrophils are implicated in the physiological turnover and/or in the increased degradation of GBM glycoproteins in vivo. We have demonstrated in both experimental models of glomerular disease that: (1) the urinary excretion of neutral proteinases is

Table 1. Effect of potential inhibitors on urinary neutral proteinase activity

Agent	Activity (%)		
	Normal rats	A rats[b]	B rats[b]
None	100	100	100
EDTA, *2 mM*	100	51.6 ± 2.4	59.1 ± 5.2
Cysteine, *2 mM*	100	52.8 ± 1.2	49.8 ± 2.6
SBTI, *500 μg/ml*	22.4 ± 2.8[a]	49.8 ± 3.9	48.6 ± 3.9
PMS-F, *1 mM*	15.1 ± 4.1	47.1 ± 2.8	56.2 ± 4.2
Aprotinin, *1 mg/ml*	19.2 ± 1.4	51.8 ± 3.6	52.3 ± 2.9
EDTA, *2 mM and PMS-F, 1 mM*	13.8 ± 2.6	9.1 ± 0.4	10.1 ± 0.6

[a] Enzymatic activity is expressed as a percentage of activity in the absence of inhibitors. Data are given as mean ± SEM ($N = 4$)

[b] A rats, rats injected with anti-GBM IgG; B rats, rats injected with aminonucleoside

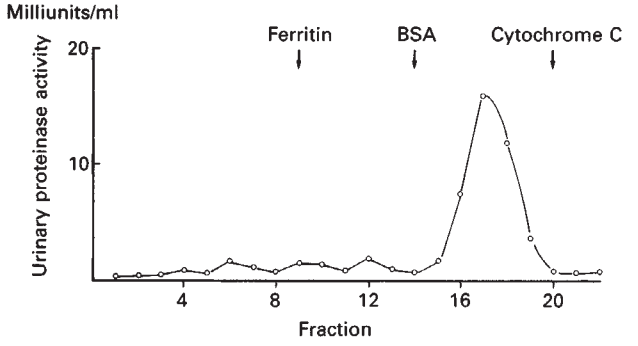

Fig. 7. *Determination of the apparent molecular weight of urinary proteinases by gel chromatography on a 1.6 × 60 cm Sephacryl S-200 SF column calibrated with ferritin, bovine serum albumine, and cytochrome C.* Fractions of 2 ml were collected and tested for proteinase activity. The proteinase activity peak exhibits an apparent molecular weight of about 30,000.

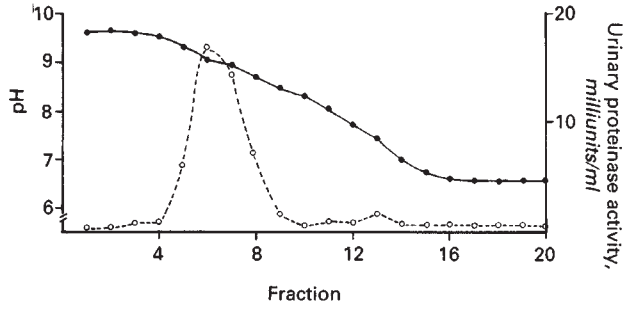

Fig. 8. *Determination of isoelectric point of urinary proteinases by chromatofocusing on a 1.6 × 40 cm column packed with PBE 94 gel.* The start buffer was 0.025 M ethanolamine—CH_3 COOH pH 9.4, whereas the eluent used was a polybuffer 96—CH_3 COOH pH 6. Fractions of 2 ml were collected, exhaustively dialyzed against 0.01 M phosphate-buffered saline pH 7.4 and tested for proteinase activity. The greatest enzymatic activity (O---O) was observed in a fraction eluted at pH (●——●) 9.0.

strongly increased; (2) these neutral proteinases are "active" since they are capable of degrading laminin and type IV collagen in vitro; (3) the urinary excretion of those enzymes is significantly correlated with the proteinuria and with the urinary

Table 2. Degradation of laminin and type IV collagen by urine from normal or nephrotic rats

Origin of urine	Degradation in 48 hr, %[a]	
	^{14}C-labelled type IV collagen	^{3}H-laminin
Normal	15.1 ± 3.2	21.6 ± 2.9
A rats[b]	52.6 ± 4.8	67.7 ± 3.8
B rats[b]	54.4 ± 3.6	69.6 ± 4.1
Control urine[b]	3.4 ± 1.6	4.7 ± 1.9

[a] Results are expressed as the percentage of laminin or of type IV collagen content of control urines. Data are given as the mean ± SEM ($N = 4$). The percentage of degradation of laminin or of type IV collagen is higher in A or B rats than in normal rats ($P < 0.01$).

[b] A rats, rats injected with anti-GBM IgG; B rats, rats injected with aminonucleoside; control urine, urine samples supplemented with PMS-F 1 mM and EDTA 2 mM

Table 3. Correlation between the urinary neutral proteinase activity and the intensity of stain for glomerular polyanion at varying time intervals in four normal rats, four rats injected with nephrotoxic serum (A rats) and four rats injected with aminonucleoside (B rats)

Group of rats	Days after injection	Urine protein *mg/24 hr*	Urinary neutral proteinase activity *U/24 hr*	Intensity of glomerular staining with colloidal iron *pH 1.9*
Control	1	5	3.6	3 +
A	1	7	2.7	3 +
B	1	8	6.1	3 +
Control	3	9	6.4	3 +
A	3	164	32.1	Trace
B	3	10	8.9	3 +
Control	4	10	5.2	3 +
A	4	172	62.2	Trace
B	10	138	45.6	Trace
Control	5	8	6.7	3 +
A	5	107	24.1	Trace
B	11	129	37.2	Trace

excretion of laminin and type IV collagen; and (4) urine from diseased rats is capable of degrading to a greater extent that normal urine labelled laminin and type IV collagen in vitro. Although the association between the proteinuria and the enhanced urinary excretion of laminin, type IV collagen and neutral proteinases may simply be coincidental, our data also support the concept [16, 19, 42] that neutral proteinases may play a role in the damage to the GBM.

We have noted that proteinases found in urine from normal or nephrotic rats are neutral proteinases since they exert their optimal activity at pH 7.4. Furthermore, inhibition experiments have shown that, in normal rats, those enzymatic activities mainly result from serine-type proteinases (inhibition by PMS-F, SBTI and aprotinin, but no inhibition by EDTA and cysteine) whereas, in proteinuric rats, they mainly result from both serine-type proteinases and metalloproteinases (inhibition by PMS-F, SBTI, aprotinin, EDTA and cysteine).

What is the origin of neutral proteinases found in urine from nephrotic rats? A bacterial contamination is highly improbable since urine samples from normal and nephrotic rats are similarly processed and sterilized by filtration through Millipore

filters before being stored at −20°C. Although they may not necessarily be the same in the two groups of rats studied, the following sources of urinary proteinases must be considered: the plasma, the phagocytic cells infiltrating the glomeruli and the intrinsic kidney cells. It is well known that the plasma contains serine–type proteinases. Besides, a metalloproteinase has been also recently detected in patients presenting with acute and chronic renal failure [43]. A role for plasma proteinases in the degradation of GBM is therefore possible despite the fact that their action is theoretically counteracted by the presence of strong plasma enzyme inhibitors [20]. Indeed, Mayer et al [44] have demonstrated that during the aminonucleoside nephrosis in rat, no change occurred in the serum protease activity whereas the serum antiprotease capacity declined coincidently with proteinuria. A relative excess of enzymes over inhibitors might then occur in the vicinity of the GBM. The contribution of such a mechanism to glomerular damage cannot be appreciated in the present study, since both protease and antiprotease activities were not measured in the serum of nephrotic rats. Polymorphonuclear leukocytes and mononuclear phagocytes are responsible for glomerular injury in animals with various immunologically induced glomerulonephritis by the selective release of lysosomal enzymes directly on the GBM [18–20, 45]. Furthermore, the aminonucleoside of puromycin can induce an in vivo release of proteases from leukocytes [46]. The morphological aspect of glomerular lesions, however, suggests that the phagocytic cells are much more probably implicated in A rats than in B rats. In this latter group indeed, no phagocytic cells are detectable in glomeruli during the course of the disease [26], whereas in A rats, some mononuclear phagocytes infiltrating the glomeruli two to five days after the injection of anti-GBM IgG are in close contact with the GBM [18]. It seems, therefore, reasonable to suggest that serine–type proteinases locally secreted by those cells may contribute to glomerular damage in rats presenting with a proliferative glomerular disease. On the contrary, a major role for mononuclear cells in the production of neutral metalloproteinases can be excluded since they can only release serine–type proteinases [20]. Finally, other possible sources of neutral proteinases are represented by intrinsic glomerular and tubular cells, which are well supplied with lysosomal hydrolases [44, 47]. It has been shown indeed that: (a) lysosomes are dramatically increased in both glomerular and proximal tubular cells during the aminonucleoside nephrosis [26]; (b) both glomerular and tubular lesions are obvious during the accelerated model of nephrotoxic nephritis three to 14 days after the injection of anti-GBM IgG [18]; (c) the mesangial cells can produce in vitro metalloproteinases capable of degrading, at physiological pH, purified rat GBM [16].

In summary, the experiments described in this paper were conducted to provide a support to the concept that neutral proteinases may contribute to GBM damage in rats presenting with a proliferative or a non-proliferative glomerular disease. Our in vivo and in vitro findings have shown that the proteinuria, the urinary excretion of basement membrane components and the urinary excretion of neutral proteinases are significantly correlated in both experimental models. It is therefore possible that metalloproteinases and/or serine–type proteinases from different sources may play a role in the development of proteinuria by their direct action on the glomerular filtration barrier.

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